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Cultivation of *Rhodotorula Toruloides* Using Palm Oil Mill Effluent: Effect on the Growth, Lipid Production, and Waste Removal

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ABSTRACT

Using Palm Oil Mill Effluent (POME) as a medium for culturing oleaginous yeast is advantageous for simultaneous lipid production and waste removal. The organic compounds in POME can be utilised as a nutrient source for yeast growth. *Rhodotorula toruloides* yeast was cultivated in filtered and unfiltered raw POME as growth media in this study. The yeast growth, pH changes in media, lipid production and removal of chemical oxygen demand (COD) of *Rhodotorula toruloides* cultivated in POME were examined and compared to *Rhodotorula toruloides* grown in yeast peptone dextrose (YPD) control media. The COD level of filtered POME was reduced by nearly 50% after filtration. The biomass concentration of *Rhodotorula toruloides* in filtered POME surpassed the other media in the following order: filtered POME > YPD > unfiltered POME (152 mg/ml > 121 mg/ml > 37 mg/ml). The filtered POME was found favourable for yeast growth due to the minimal amount of colloidal particles and suspended solids. Meanwhile, the lipid production (4.51 %) in filtered POME was 4.8-fold higher than in control media. The water analysis indicated about 43% of COD reduction, signifying the ability of *Rhodotorula toruloides* to utilise nutrient components present in POME for growth. This study provides insightful knowledge on the utilisation of oleaginous yeast for simultaneous green waste disposal and sustainable microbial oil production.

Keywords: Chemical oxygen demand, lipid production, palm oil mill effluent, *Rhodotorula toruloides*, waste treatment

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INTRODUCTION

The palm oil industry is an important commodity and the largest agriculture-based industry in Malaysia (Chin et al., 2013). Compared to other countries, Malaysia is

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rich in tropical forests and agricultural vegetation, which has led to the industry's success, which sought the growth of palm trees (Foo & Hameed, 2010). The increased demand for palm oil has contributed to palm oil production worldwide. Palm oil production is anticipated to increase from 72.27 million metric tonnes in 2019/2020 to 73.23 million metric tonnes in 2020/2021 (Shahbandeh, 2021). Palm oil industries contributed about 5% to 7% of Malaysia's gross domestic product (GDP) (Lee et al., 2020). The rapid production of palm oil, however, generates an abundance of wastes such as empty fruit bunches, mesocarp fibre, palm kernel shells, oil palm frond, oil palm trunk, and palm oil mill effluents (POME) (Lee et al., 2020).

Concerns about the detrimental environmental effect of the massive amount of palm oil-based wastes have been raised. Raw POME contains high chemical oxygen demand (16,000–100,000 mg/L), biochemical oxygen demand (10,250–43,750 mg/L), total suspended solids (5,000–54,000 mg/L), oils and greases, and ammoniacal nitrogen with highly acidic pH determined around 3 to 4 (Abdulsalam et al., 2018). The presence of these contaminants makes the disposal of POME a pollution issue. Therefore, further research into developing alternative approaches for POME treatment becomes crucial in combating the environmental impact.

According to Amat et al. (2015), there are few known conventional methods for treating POME, including evaporation, adsorption, and advanced oxidation (ozonation technique), which primarily involves decolourising the POME from its thick brownish colour into the colour of pure, clean water. Out of the many alternatives methods explored for POME treatment, previous findings showed that aerobic and anaerobic biological processes have significantly catered to energy efficiency, low nutrient intake, and biomass yield (Cheng et al., 2010).

More than 85% of POME producers in Malaysia employ ponding systems with anaerobic technology for POME treatment due to the low cost of operation and capital (Abdurahman et al., 2011). Nonetheless, this approach has surged issues threatening the environment due to extensive land usage and long retention time (Abdurahman et al., 2011). Therefore, it indicates that further approaches for a green and cost-effective POME treatment should be explored.

The employment of microbial oils on POME as cheap media is beneficial for green waste management and essential for the cost-effective production of valuable products (Marjakangas et al., 2015). Oleaginous microorganisms, such as yeast, microalgae, fungi, and bacteria, can produce more than 20–25% microbial lipids or single-cell oils like vegetable oils composition (Liang & Jiang, 2013). The application of oleaginous microorganisms in treating POME has gained widespread acceptance as a potential replacement for vegetable oils and future biodiesel. Culturing oleaginous microorganisms is more practical since it is unaffected by seasons or climate change (Cheirsilp & Louhasakul, 2013).

Oleaginous yeasts are attractive due to their ability to use low-cost fermentation media, rapid growth, and feasibility for large-scale cultivation (Cheirsilp & Louhasakul, 2013). *Yarrowia sp.*, *Candida sp.*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus sp.*, *Trichosporon sp.*, and *Lipomyces sp.* are lipid-producing yeasts (Marjakangas et al., 2015). *Rhodosporidium sp*., *Rhodotorula sp*., and *Lipomyces sp*. can accumulate intracellular lipids up to 70% of their dry cell mass (Li et al., 2007). Marjakangas et al. (2015) proposed utilising oleaginous yeast, which is naturally present in POME for lipid production and COD removal, as wastewater sterilisation in large-scale operation is not economically practicable. Their study achieved 94.5% COD removal using oleaginous yeast *Graphium penicillioides* grown in POME, with 29.1% (w/w) lipid production.

According to Islam et al. (2018), yeast could take up minerals and vitamins from POME to stimulate cell growth and product formation. *Rhodotorula toruloides* of the *Pucciniomycotina* subphylum exhibited an unprecedented rate of high cell density fermentation, high carotenoid, and lipid productivity using cheap substrates as growth medium (Liu et al., 2018). Furthermore, *Rhodotorula toruloides* showed COD reduction in wastewater by 72.3% using the feeding mode strategy (Qin et al., 2017). Arous et al. (2019) demonstrated that POME bioremediation by yeast has contributed to a COD removal of about 90%. Meanwhile, Karim et al. (2021) demonstrated an enhanced lipid accumulation and COD removal of approximately 80% using a mixed culture of yeast and bacteria. Nevertheless, there has been limited study on the growth behaviour of *Rhodotorula toruloides* on raw POME media for lipid and biomass production.

A local oleaginous yeast *Rhodotorula toruloides* has been successfully isolated from an acidic environment from a copper mining site. Its growth behaviour on POME was examined in this study. The aim was to evaluate the potential of the yeast to reduce the COD levels of POME while simultaneously producing lipid as a valuable product. The results were compared with the yeast growth cultivated in yeast peptone dextrose (YPD) media as a controlled study. The resulting lipid production and COD removal of the yeast were further evaluated. The findings of this study will lead to the utilisation of oleaginous yeast for a cost-effective POME treatment as part of bioremediation and simultaneously provide sustainable microbial oil production.

MATERIALS AND METHODS

Microorganism

Oleaginous yeast *Rhodotorula toruloides* was locally isolated from runoff water of Ranau River, Sabah (6.030324°; 116.657833°).

Preparation of POME Media

Palm oil mill effluent (POME) was collected from Langkon Palm Oil Mill, Kota Marudu, Sabah (6.57098°; 116.70748°). The POME samples were placed into a 250-ml conical flask and then filtration through Whatman filter paper (0.2 nm) to remove mud and large particles before yeast cultivation. Unfiltered POME samples, which did not undergo any filtration process, were used as a comparison study for yeast cultivation. *Rhodotorula toruloides* were grown on a yeast peptone dextrose (YPD) agar plate and incubated for 24 hours at 30 °C. The cultures were then inoculated in a 250 ml Erlenmeyer flask containing 150 ml sterilised media (filtered POME, unfiltered POME, or YPD media) and incubated at 30 °C on an incubator shaker (New Brunswick Scientific) at 180 rpm for 24 hours for preparation of inoculum. Every new experiment was carried out using fresh culture from the inoculum.

Cultivation of Yeast in POME Media

Approximately 150 ml of filtered POME, unfiltered POME, and YPD media were prepared in 250 ml conical flasks. The initial pH of the media was adjusted to 6.0 and sterilised by autoclaving at 121 °C for 20 minutes before use. The media was inoculated with 10% (v/v) of a mid-log phase *Rhodotorula toruloides* inoculum (approximately OD_{600} = 1.8–2.2) and incubated at room 30 °C on an incubator shaker at 180 rpm for 72 hours. Samples were harvested every 2 hours interval for biomass determination to determine the growth behaviour of the yeast on POME. The pH changes in the cultivation media were also observed and determined using a pH meter (Professional Benchtop pH Meter BP3001, Trans Instruments, Singapore). Lipid production and COD content of the waste media were determined after 36 hours of the cultivation of *Rhodotorula toruloides*.

Characterisation of POME

The physicochemical properties of the filtered and unfiltered POME as a culture medium for *Rhodotorula toruloides* were analysed at the Water Research Unit, Faculty of Science and Natural Resources, Universiti Malaysia Sabah. The samples were analysed using the DOE method (Department of Environment, 2011) concerning Revised Standard Methods (1985). The tested water quality analyses include the biochemical oxygen demand (BOD), chemical oxygen demand (COD), total Kjedahl nitrogen, total suspended solids, oil, grease, ammoniacal nitrogen, and orthophosphate.

Determination of Biomass Concentration

The harvested samples were placed in a 50-ml falcon tube, spun down at 3,400 x g for 5 minutes and washed with deionised distilled water. After that, the samples were dried at 60 °C temperature until constant weight (Kitcha & Cheirsilp, 2011). Biomass concentration was measured gravimetrically every two hours using Equation 1 (Nascimento et al., 2013).

Biomass concentration (mg/ml) = (x2 – x1) (1)

Where x2 was the weight of the centrifuge tube together with biomass after drying, and x1 was the weight of the centrifuge tube.

Determination of Lipid Content

The harvested biomass samples were centrifuged, dried, and grounded into powder biomass. Lipid content in the sample was determined using Bligh and Dyer's (1959) method, whereby a 1:1:1 mixture of chloroform, methanol, and distilled water were added into the pre-weighed dried biomass samples inside a falcon tube. The mixtures were vortexed and left on the bench for 1 hour. Next, the samples were centrifuged at 5,500 x *g* for 5 min, resulting in the formation of two layers. The top layer (methanol and water) was discarded, while the bottom layer (chloroform and lipid) was transferred into a pre-weighed microcentrifuge tube. Speed-vacuum (V-AQ) was used for 1.5 hours to complete methanol removal. Finally, the extracted lipid was weighed, and the lipid content was calculated using Equation 2 (Cordeiro et al., 2017).

Lipid content (%) = [extracted lipid extract (g) / dry biomass (g)] × 100 (2)

Determination of COD Removal

The COD content of POME samples was determined before and after treatment with *Rhodotorula toruloides.* The samples were analysed using the DOE method (Department of Environment, 2011) with reference to Revised Standard Methods (1985) at the Water Research Unit, Faculty of Science and Natural Resources, University Malaysia Sabah. The removal of COD was determined using Equation 3.

$$COD\ removal\ (\%) = \frac{COD_{untreated\ POME} - COD_{treated\ POME}}{COD_{untreated\ POME}} \quad (3)$$

Statistical Analysis

All experiments were carried out in triplicate. The data were analysed using the statistical tool in MS Excel 2010 and presented as a mean value with an average standard deviation (SD) of < 5%.

RESULT AND DISCUSSION

Characteristics of POME

The collected POME sample was pre-filtered to remove mud and solid wastes. Its physicochemical properties were determined and compared to the unfiltered POME sample

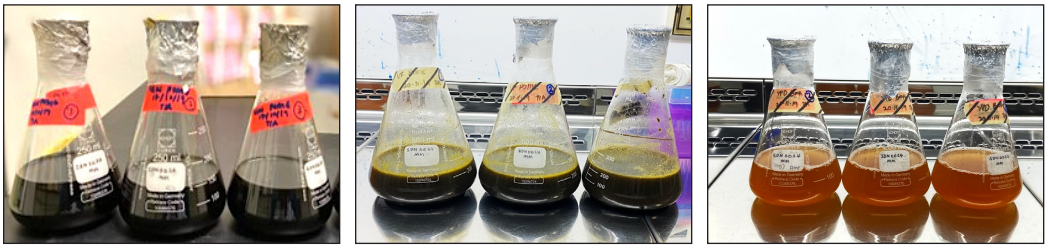
as tabulated in Table 1. The unfiltered POME was found very thick, dark brownish, and odorous. The filtered POME showed a cleaner look (Figure 1a) and was free from mud and solid wastes present in the unfiltered POME (Figure 1b). The unfiltered POME may contain colloidal suspensions, including water, oil, and suspended solids from palm fruit residues, as Kaman et al. (2016) described. The BOD and COD content of the unfiltered POME was determined to be about 50,750 mg/L of BOD and 68,031 mg/L. After the filtration process, the BOD and COD values of POME were largely reduced to 70% and 50%, respectively. This finding parallels the study Lokman et al. (2019) reported on the successful reduction of BOD concentration by approximately 50% to 65% after the filtration process. The total Kjeldahl nitrogen in the unfiltered POME exceeded 30,000 mg/L, while the ammoniacal nitrogen was about 11 365 mg/L. It is important to note that total nitrogen and ammoniacal nitrogen content dropped by about 99% upon filtration. A similar observation was reported by Sajjad et al. (2018) on the removal of ammoniacal nitrogen of about 99% using a combination of biofilm and nano-filtration membrane. Both filtered and unfiltered POME were further evaluated for yeast growth and lipid production.

Table 1
Characteristics of POME

Parameters	Unit	Unfiltered	Filtered
Biochemical oxygen demand	mg/L	50, 750	15, 275
Chemical oxygen demand	mg/L	68, 031	33, 923
Total Kjeldahl nitrogen	mg/L	30, 845	280
Ammoniacal nitrogen	mg/L	11, 365	74
Orthophosphate	mg/L	23.20	18.6
Total suspended solids	mg/L	23, 244	136
Oil and grease	mg/L	16, 188	8

Growth Profile of *Rhodotorula toruloides*

Rhodotorula toruloides yeast was grown in filtered (Figure 1a), and unfiltered POME (Figure 1b) medium, and their growth characteristic were benchmarked to yeast cultured on YPD defined media (Figure 1c). The POME samples were darker than the YPD media as



(a) (b) (c)

Figure 1. Cultivation of *Rhodotorula toruloides* in (a) filtered POME, (b) unfiltered POME, and (c) YPD broth

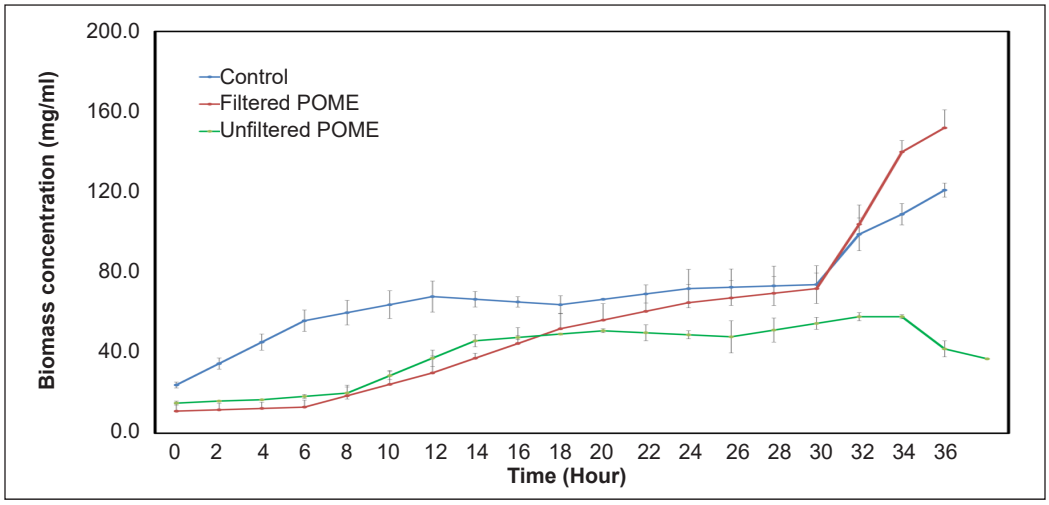


Figure 2. The growth profile of *Rhodotorula toruloides* in filtered POME, unfiltered POME, and YPD media

they contained high organic compounds. Figure 2 shows the growth profile of *Rhodotorula toruloides* in POME and YPD medium as a function of biomass concentration over time. As can be observed, the growth of *Rhodotorula toruloides* in filtered POME media resulted in a maximum biomass concentration of 152.0 mg/ml at 36 hours, as opposed to YPD media (121.0 mg/ml) and unfiltered POME media (37.0 mg/ml).

According to the findings, the yeast growth in filtered media achieved the highest growth compared to control and unfiltered POME media, 152 mg/ml > 121 mg/ml > 37 mg/ml, respectively. According to Hahn et al. (2005), microorganisms have grown more aggressively in rich, complex media than in defined media due to biosynthetic precursors, which can be transported directly into anabolic pathways and aid in reducing the production of biosynthetic precursors while saving metabolic energy. From 30 to 36 hours, both control and filtered POME medium showed a fast surge in growth, whereas the culture in unfiltered POME displayed an early death phase trend. The sudden rise in concentration from 30 to 36 hours, from 74 to 121 mg/ml, was most likely induced by the nutrient available in the media, particularly carbon source, which regulates the biosynthesis of internal lipids in yeast cells (Kot et al., 2019).

As for the cultivation of *Rhodotorula toruloides* in unfiltered POME, the biomass concentration did not exceed the biomass yield in the control media. The enormous quantity of colloidal particles, suspended solids, and bacteria inside the unfiltered POME, as indicated in Table 1, made such an event possible by impeding yeast development. The physical form of the unfiltered POME has most likely hindered cell mobility in search of food sources. The observation was seen here also corroborates the findings of earlier reported studies. Soleimaninanadegani et al. (2014) described the growth inhibition as due to the colloidal and suspended components floating on wastewater surface, obstructing the

respiration process of the microorganisms. Furthermore, Bala et al. (2018) showed that the existence of indigenous bacteria in POME could result in a food competition. The cells' growth dropped drastically after 32 hours of cultivation from 58.0 mg/ml to 37.0 mg/ml, implying the beginning of the death phase.

These growth trends of *Rhodotorula toruloides* demonstrated its ability to utilise carbon, nitrogen, and other nutrient sources in POME. It signifies the great potential of agricultural wastes as growth promoting material for microorganisms cultivation. Other studies have also described waste as growth media as a new technique for long-term agricultural development. By combining food waste with biochar obtained from oil palm trash, sugarcane, and sawdust, Ma et al. (2019) revealed the potential of reusing diaper waste as an orchid growing substrate. A unique combination of used diapers and other food wastes, such as banana peels and eggshells, has also been demonstrated to be a green method for mushroom cultivation with no accumulation of undesired toxic products (Ma et al., 2020, Nam et al., 2018).

pH Changes of *Rhodotorula toruloides*

The pH of the culture media was monitored. As shown in Figure 3, the pH of filtered POME has swiftly shifted from acidic to alkali compared to the control media. It is observed that during the early hours of yeast cultivation, from 0 to 6 hours, the pH value of filtered and unfiltered POME dropped from pH 6.0 to 5.6 and 5.7, respectively. Meanwhile, the pH value of control media remained constant throughout yeast cultivation. Nevertheless, filtered POME media showed a rapid increase in pH from 5.6 to 7.1 up to 34 hours of cultivation, then a slight decrease to 6.8 at hour 36. Unfiltered POME media followed the same pattern, although not as quickly as filtered POME media.

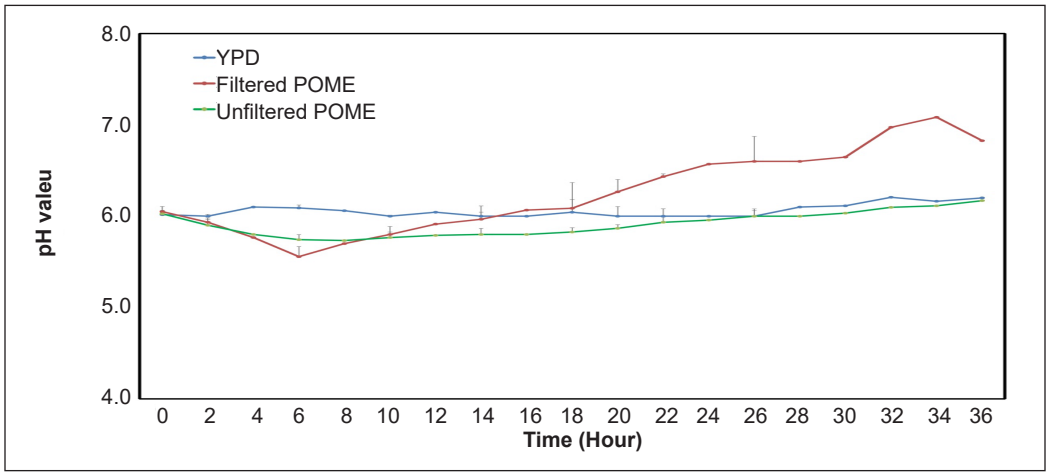


Figure 3. pH changes of media during cultivation of *Rhodotorula toruloides* in filtered POME, unfiltered POME, and YPD media

The pH reduction in filtered POME indicated that glucose was consumed in the media, resulting in the formation of acid chemicals by the yeast, as reported by Yu et al. (2018). Due to glucose depletion and the yeast's inability to produce acids, the pH rapidly rose after 6 hours of cultivation. The slight decline of pH in filtered POME after 36 hours of cultivation could be due to the lactic acid fermentation by the existing bacteria in the wastewater media (Gientka et al., 2017), as evidenced by the rapid increase in biomass concentration at 36 hours (Figure 2).

According to Gientka et al. (2017), the growth development of yeast is favoured in a slightly acidic environment, whereas an alkalisation environment may induce stress on the yeast. On the other hand, the yeast in this study yielded a higher biomass concentration as the pH increased. It might be due to the varied types of media and yeast strain employed, which could trigger yeast growth rate and pH changes (Jiru et al., 2017).

Lipid Production of *Rhodotorula toruloides*

As the growth of *Rhodotorula toruloides* was greater in filtered POME, the lipid production and COD removal ability of the yeast were further investigated in this media. Figure 4 depicts the lipid content of yeast grown in filtered POME and YPD media for 36 hours of cultivation. *Rhodotorula toruloides* grown in the POME medium had a greater lipid content (4.51%) than those produced in control media (0.94%). The yeast cultured in the POME medium had a high lipid content, corresponding to the high biomass yield depicted in Figure 3. Lipid formation in oleaginous bacteria normally occurs during secondary metabolic growth in a carbon excess and nitrogen limitation environment (Matsakas et al., 2017). The lipid accumulation was triggered by assimilating the access substrate and converted to fat for storage (Li et al., 2007). An earlier study reported *Rhodotorula glutinis* lipid production up to 21% (w/w) grown in POME (Leiva et al., 2014). Oleaginous yeast *Lipomyces starkeyi,* on the other hand, generated approximately 7.4% (w/w) lipid content and enhanced the lipid production to 21.3% (w/w) by diluting POME with deionised water in a 1:1 ratio (Islam et al., 2018). The presence of a high number of inhibitors in undiluted POME hindered the lipid production and growth rate of *L. starkeyi*. The cultivation of microalgae *Chlorella* sp on POME also

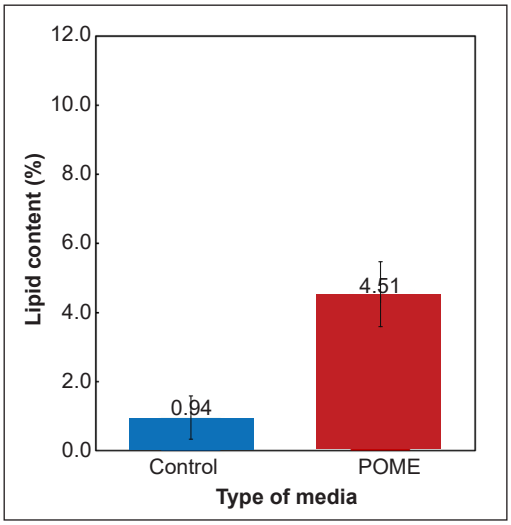


Figure 4. Lipid production of *Rhodotorula toruloides* grown in YPD media and filtered POME for 36 hours of cultivation at 30°C

agreed that enhanced lipid production could be obtained using diluted POME media (Kamyab et al., 2017). The lipid production of *Rhodotorula toruloides* probably can be enhanced further in future by optimising the concentration of POME.

COD Removal of *Rhodotorula toruloides*

Figure 5 depicts the COD values of POME samples before and after *Rhodotorula toruloides* cultivation in the POME. The COD content of untreated POME was determined at 33 923 mg/L. After the treatment, the COD value was significantly reduced to 19 290 mg/L. The data shows that the COD removal was about 43%. Based on the result, yeast has significantly utilised the nutrients present in POME during the biological process, such as respiration, for its growth. *Rhodotorula toruloides* converted the organic component found in POME into lipids, decreasing the COD level. A previous study showed that the cultivation of *Yarrowia lipolytica* on POME achieved approximately 88.4% of COD removal (Louhasakul et al., 2020), about 45% higher than the present study. It was due to the addition of a biosurfactant that aided the cells in utilising the carbon compounds, specifically the hydrocarbons compounds in POME (Louhasakul et al., 2020). Thus, this explained the small amount of COD reduction in this current study due to the application of pure POME.

Higher COD removal (72–95%) was reported using a 50% diluted POME concentration. Islam et al. (2018) found that 50% diluted POME supports a greater growth rate of oleaginous yeast due to fewer phenolic compounds present in POME, resulting in increased COD reduction. As yeast thrives in nitrogen-limited circumstances, a high quantity of phenolic compounds may impede yeast growth and metabolic activity inside the POME owing to a high ammonium concentration (Islam et al., 2018). According to Saenge et al. (2011), COD removal can also be enhanced by 65.1% by adding nitrogen sources like yeast extract into POME. Even though yeast extract is a good nitrogen source, its high cost makes it uneconomical for industrial operations. *Rhodotorula sp*. could convert organic compounds in POME to lipid and carotenoids, which is beneficial for COD reduction (Saenge et al., 2011).

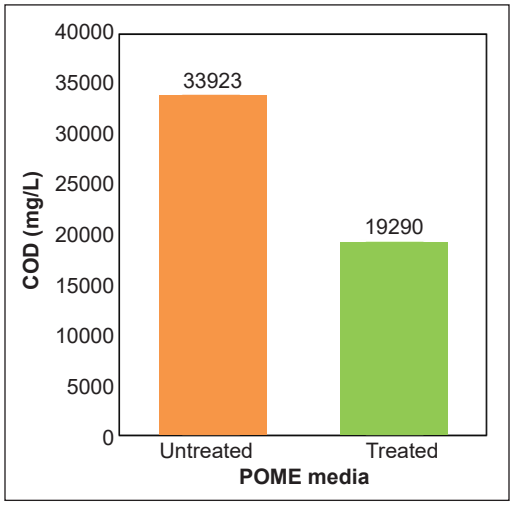


Figure 5. COD values of filtered POME before and after the cultivation of *Rhodotorula toruloides*

Rhodotorula toruloides grew best when cultivated in filtered POME with a 152 mg/ml biomass concentration and a lipid content of 4.51%. This study shows the ability of the yeast to utilise the nutrients present in POME for growth. The filtered POME

sample was found favourable for the cultivation of *Rhodotorula toruloides* as growth media. However, suspended solids and other colloidal particles in unfiltered POME may reduce the accessibility of available nutrients for yeast growth. By benchmarking the findings with past reported studies, as summarised in Table 2, this study shows that *Rhodotorula toruloides* grown in filtered POME were comparable to or even better than the reported oleaginous microorganisms cultivated in various cheap media for lipid production and COD removal. The study's findings provide beneficial insight into utilising agricultural wastes as cheap media for practical industrial applications.

Table 2
Comparative study of various types of microorganisms cultivated in different types of media based on their lipid content and COD removal efficiency

Microorganisms	Types of media	Lipid content % (w/w)	COD removal (%)	Reference
Rhodotorula toruloides	POME	4.51	43.0	This study
D. etchellsii BM 1	62.4% expired soft drinks + 37.6% olive mill wastewater	28.1	41.3	Arous et al. (2016)
C. tropicalis	Olive mill wastewater + 132 C/N ratio	78.7	13.9	Dias et al. (2021)
C. tropicalis	Olive mill wastewater + Urea	13.3	36.3	Dias et al. (2021)
T. coremiiforme	acetone-butanol-ethanol fermentation wastewater + oleic acid (C16:1)	1.06	64.14	Xiong et al. (2016)
T. cutaneum	acetone-butanol-ethanol fermentation wastewater + oleic acid (C16:1)	0.77	69.46	Xiong et al. (2016)
Rhodotorula toruloides	Cellulosic ethanol fermentation wastewater	17.32	38.72	Yao et al. (2020)

CONCLUSION

The efficiency of POME as growth media for *Rhodotorula toruloides* was evaluated and compared with yeast standard media. As evident by the characteristic analysis and growth profile of yeast, the reduced number of pollutants available in POME was found favourable for the yeast growth. Filtered POME enhanced biomass production about 4.1 times in comparison with non-filtered POME. The cultivation of *Rhodotorula toruloides* on POME has successfully reduced COD content by about 43 % and simultaneously increased the lipid production by 1.8-fold. The findings of this study indicate that POME can be used as a cost-effective substrate for the cultivation of *Rhodotorula toruloides*, which would be beneficial for waste treatment and biofuel production. For future study, *Rhodotorula toruloides* can be co-cultivated with other oleaginous microorganisms for enhanced lipid production and waste removal.

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